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Capsular Polysaccharide of *Clostridium perfringens* Hobbs 10

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A capsular polysaccharide was isolated from a strain of *Clostridium perfringens* Hobbs 10 type A by cold-water extraction of whole, heavily encapsulated cells. The water-soluble polymer was isolated by alcohol precipitation and purified by treatment with chloroform-butanol, cetytrimethylammonium bromide, and column gel permeation chromatography by using Bio-Gel A-5m agarose. The formation of a single precipitin line, when the isolated polysaccharide was reacted with its homologous antisera by double diffusion in gel, was considered a criterion of immunochemical purity. The purified polymer appeared as a single peak when eluted from diethylaminoethyl-Sephadex with a linear gradient of NaCl. The polysaccharide was composed of glucose, galactose, galactosamine, and iduronic acid in a molar ratio of 4.1:5.1.7:1, respectively. These constituents accounted for 83% of the dry weight. The polysaccharide appeared to have a molecular weight of 40,000 and exhibited aggregation up to 120,000. A trace of peptide material could not be removed during purification.

Early attempts to classify *Clostridium perfringens* established the immunological relationships of the four main groups (A through D) based on the presence of three lethal toxins (22). The subsequent identification of 12 different toxic elements led to the grouping of *C. perfringens* into five types (A through E) according to toxin production (22). *C. perfringens* has eluded further subdivision into biotypes primarily because of somatic and capsular antigen heterogeneity (18, 22), the diversity of antigen preparation methods, and lack of definitive chemical characterization.

Hobbs et al. (13, 14), Smith and Holdeman (18), and Willis (22) suggested a scheme of strain differentiation by provisionally designating serological types 1 through 8 from a group of 11 selected strains. The complexity of the capsular antigens in this species is illustrated by the results of the Anaerobic Bacteriology Laboratory, National Center for Disease Control, Atlanta, Ga. (9), which has prepared 74 distinct antisera against as many strains of *C. perfringens* in addition to the 13 serotypes classified by Hobbs. Baine and Cherniak (2) characterized a type-specific capsular polysaccharide obtained from *C. perfringens* Hobbs 5. In a later study, Cherniak and Henderson demonstrated that each of four strains of *C. perfringens* investigated contained a capsular polysaccharide which was strain specific (6). Data for the presence of a group-specific antigen was also described (6).

The purpose of this study was the isolation and partial chemical and immunochemical characterization of the homogeneous type-specific polysaccharide of *C. perfringens* Hobbs 10. The characteristic properties of the capsular polysaccharides determined in this and similar studies will permit the comparison of *C. perfringens* strains at the molecular level.

(This paper is taken in part from a thesis presented by Linda Lee to the Graduate School of Arts and Sciences of Georgia State University in partial fulfillment of the requirements of the M.Sc. degree.)

MATERIALS AND METHODS

Culture and antisera. A culture of *C. perfringens* Hobbs 10 and specific rabbit antisera, prepared with formalinized Hobbs 5 and Hobbs 10 cells, were a gift of V. R. Dowell, National Center for Disease Control, Atlanta, Ga. The encapsulated strain of *C. perfringens* was selected, maintained, and isolated as previously described (2).

Immunodiffusion. Immunological specificity was determined with rabbit antisera against *C. perfringens* Hobbs 5 and 10 by a technique of double diffusion in gel reported previously (6).

Molecular weight. Molecular weights were determined by the meniscus-depletion sedimentation equilibrium technique of Yphantis (23) and Roark and Yphantis (16). All experiments were performed in 1 M NaCl, 25 C, at 16,000 rpm. Interference photographs were analyzed to yield plots of weight-average molecular weights as a function of local equilibrium concentration, and were analyzed by two-species plots to assess heterogeneity and monomer molecular weights.

Analytical procedures. The determination of neutral carbohydrate was accomplished by the phenol-sulfuric acid method of DuBois et al. (10). The glucose and galactose contents of the polysaccharide fractions were assayed with glucostat and galactostat reagents (Worthington Biochemical Corp.) after hydrolysis for 50 min in 2 N HCl at 100 C. Hexosamine was quantitated after hydrolysis of the sample with 2 N HCl for 18 h at 100 C by the method of Elson and Morgan (11) as modified by Boas (3). The identity of the hexosamine was confirmed by chromatography on a Beckman amino acid analyzer and by the ninhydrin degradation procedure of Stoffyn and Jeanloz (19). The uronic acid constituent was determined by the carbazole (8) and orcinol (4) reactions with glucuronic and iduronic acid as standards. Phosphate was estimated by the Ames and Dubin (1) modification of the method of Chen et al. (5). Protein content was ascertained with Folin-Ciocalteau reagent (12) according to Lowry et al. (15).

Carbohydrate constituents were identified qualitatively by descending chromatography on Whatman no. 1 paper, after acid hydrolysis, with the following solvent systems: (i) 1-butanol-pyridine-water (6:4:3, vol/vol); (ii) ethyl acetate-pyridine-water-acetic acid (5:5:3:1, vol/vol); and (iii) 1-butanol-acetic acid-water (10:3:1, vol/vol). Sugars were detected with alkaline silver nitrate (21) and hexosamine with ninhydrin.

Vapor phase chromatography was performed with a Beckman model GC-65 equipped with dual-flame ionization detectors. Analyses were conducted with columns (6 ft, about 1.9 m) of 3% SE 52 on 100- to 120-mesh high-performance chromosorb W and helium as carrier gas (20).

The trimethylsilyl ethers of the samples were introduced at 140 C, and this temperature was held for 7 min. The column temperature was programed to rise to a maximum of 180 C at a rate of 2.75 degrees per min after the hold interval.

Polysaccharide. One hundred grams of acetone dried cells, divided into 25-g portions, were coarsely ground with a mortar and pestle and suspended in 300 ml of water. The cells were dispersed by vigorous stirring in a boiling-water bath for 5 min. The suspension was cooled rapidly, and stirring was continued for 90 min in an ice-water bath to solubilize the polysaccharide. The soluble material was separated from the residue by centrifugation (27,000 $\times$ *g* for 30 min). The supernatant fraction was adjusted to 1% acetic acid, and the crude polysaccharide was precipitated by the addition of 2 volumes of 95% ethanol. The precipitate was collected by centrifugation (27,000 $\times$ *g* for 30 min) after allowing the suspension to stand overnight in the refrigerator. The crude polysaccharide was washed with an increasing concentration of ethanol and finally with acetone. Residual acetone was removed in vacuo, and the precipitate was stored over $CaCl_2$.

Crude polysaccharide (7.1 g) was dissolved in 550 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-0.1 M NaCl buffer, pH 7.5, and samples were extracted with equal volumes of chloroform-butanol (9:1, vol/vol) in a blendor for 10 min at low speed (17). Extraction of the aqueous layer with fresh solvent was continued until little or no denatured protein appeared at the solvent interface after centrifugation (3). The aqueous phases were combined, and the deproteinized polysaccharide was recovered by precipitation with ethanol as described above.

The deproteinized polysaccharide (3.3 g) was dissolved in 500 ml of 0.01 M Tris-0.02 M Na_2SO_4-0.2 M NaCl, pH 7.5. The nucleic acids were precipitated by adjusting the solution to 1% with respect to cetyltrimethylammonium bromide (CTAB) and allowing it to stand overnight (7). The suspension was centrifuged for 10 min at 10,000 $\times$ *g*, and the sediment was discarded. The supernatant was adjusted to 1% acetic acid, and the polysaccharide was precipitated by the addition of 2 volumes of 95% ethanol. The polysaccharide was dissolved in water, exhaustively dialyzed against distilled water, and reprecipitated several times in the usual manner with acetic acid-ethanol, and the final precipitate was washed successively with 95% ethanol, absolute ethanol, and acetone. Residual acetone was removed in vacuo, and the precipitate (CTAB supernatant) was stored over $CaCl_2$.

A portion of the CTAB supernatant (50 mg) was dissolved in 5 ml of 0.01 M Tris-1.0 M NaCl, pH 7.5, and the sample was applied to a Bio-Gel A-5m (200 to 400 mesh) agarose column (2.5 by 90 cm) which had been previously equilibrated with the same buffer. The polysaccharide fractions, eluted at a flow rate of 8 ml/h, were monitored by the phenol-sulfuric acid reaction and by ultraviolet absorbance at 260 and 280 nm. A more detailed analysis of the individual constituents of fractions was performed occasionally. Appropriate fractions obtained from several column runs were combined, dialyzed, and lyophilized. The homogeneity of the agarose fraction was tested by chromatography on a column (1.5 by 30 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 Cl. The sample was eluted with 500 ml of elutant over a linear concentration gradient of 0 to 0.5 M NaCl.

RESULTS

Purification. Cold-water treatment of *C. perfringens* Hobbs 10 followed by alcohol precipitation of the supernatant fraction yielded a crude polysaccharide corresponding to 7.5% of the cell dry weight (Table 1). Acid hydrolysis of this fraction followed by paper chromatography in solvent (i) demonstrated the probable presence of glucose, galactose, galactosamine, and ribose. An analytical comparison of the chloroform-butanol-deproteinized polysaccharide with the crude polysaccharide showed a 2.0-fold increase in hexosamine, a 1.5-fold increase in neutral sugar, and an approximately 4-fold drop in protein content (Table 1). Analysis of the CTAB supernatant showed an improvement in purity over the deproteinized sample as illustrated by an additional 1.3-fold increase in the neutral sugar value, a 1.7-fold increase in hexosamine, and a concomitant decrease in phosphate content (Table 1).

TABLE 1. *Composition of capsular polysaccharide of C. perfringens Hobbs 10*

Fraction	Neutral sugar[a]	Hexosamine	Phosphate	Protein	Recovery from 100 g of dried cells (%)
Crude polysaccharide	17.0	2.4		31.0	7.5
Deproteinized polysaccharide	25.6	4.8	12.7	7.3	3.5
CTAB supernatant	34.2	7.9	8.0	12.7	1.7
Fraction I (agarose column)	50.6	12.0	0.2	1.4	1.6

[a] Numbers equal percentage composition by dry weight.

The CTAB supernatant fraction reacted monospecifically with both *C. perfringens* Hobbs 5 and 10 antisera in double diffusion in gel experiments.

The first peak (agarose fraction I) eluted from a Bio-Gel A-5m agarose column contained virtually all of the polysaccharide, with little contamination from other substances (Fig. 1, Table 1). Analysis of agarose fraction I demonstrated a five-fold overall increase in purity, based on the hexosamine content, and the concomitant removal of the phosphate- and protein-containing substances (Table 1). Chromatography of agarose fraction I on a DEAE-Sephadex column yielded a single major carbohydrate fraction which was eluted at 0.2 M NaCl (Fig. 2).

Immunodiffusion showed that agarose fraction I reacted monospecifically with *C. perfringens* Hobbs 10 antiserum but failed to react with *C. perfringens* Hobbs 5 antiserum. Agarose fraction II, consisting of the pooled tubes between agarose fraction I and the nucleic acid peak (agarose fraction III) reacted monospecifically with both antisera. The precipitin line obtained with *C. perfringens* Hobbs 5 antiserum was eliminated by in situ cross-absorption with a fraction obtained from *C. perfringens* Hobbs 5 (peak I of Baine and Cherniak [3]).

Paper chromatography analysis of acid hydrolysates showed that agarose fraction II contained ribose with traces of glucose and galactose, whereas agarose fraction III yielded only ribose.

Identification and analysis of the carbohydrate constituents. Acid hydrolysates of agarose fraction I analyzed by paper chromatography in solvents (i) and (ii) demonstrated the presence of galactosamine. Ninhydrin degradation resulted in the formation of lyxose, as would be expected if galactosamine were the only hexosamine present. Automated amino acid analysis confirmed the presence of galactosamine as the single hexosamine component; trace amounts of amino acids, comprising less than 1% of the sample, were also detected. Quantitative analysis indicated that galactosamine represented 12% of the sample (Table 2).

The neutral sugar constituents were identified as glucose and galactose by paper chromatography in solvents (i) and (ii) and by vapor phase chromatography. Maximal glucose and galactose release was attained by hydrolysis of the test samples for 50 min in 2 N HCl. The specific enzyme reagents, glucostat and galactostat, resulted in the quantitative estimation of glucose and galactose as 29.0 and 35.2%, respectively (Table 2); in addition, the positive reactions confirmed their identity.

The uronic acid moiety gave a carbazole-to-orcinol ratio of 0.32. Iduronic acid determined in the same manner gave a ratio of 0.31. Paper chromatography in solvents (ii) and (iii) dem-

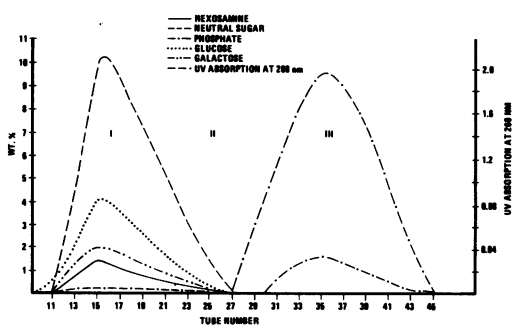

FIG. 1. *Typical elution curve of CTAB supernatant fractionated on Bio-Gel A-5m agarose.*

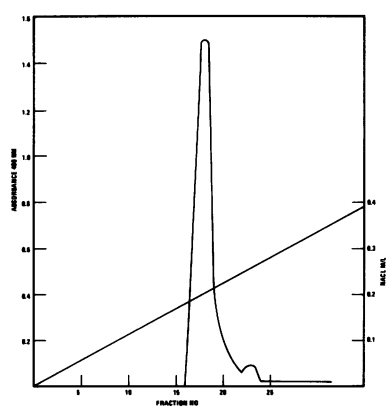

FIG. 2. *Rechromatography of fraction 1 on DEAE-Sephadex A25.*

onstrated that the mobility of the uronic acid constituent coincided with that of iduronic acid. A detailed communication confirming the tentative identification of the uronic acid constituent as iduronic acid will be reported separately (L. Lee and R. Cherniak, manuscript in preparation). The uronic acid moiety represented 7.0% of the polysaccharide (Table 2).

Molecular weight. Sedimentation equilibrium experiments determined values of the weight and Z-average molecular weights as a function of local equilibrium concentration. Presentation of the averages by the two-species method of Roark and Yphantis (16) indicated that the polysaccharide was predominantly 40,000 daltons, with significant amounts of higher aggregates up to at least 120,000 daltons.

DISCUSSION

The low tenacity of type-specific polysaccharide exhibited for the cell surface was demonstrated by a drastic decrease in the viscosity of cultures grown with moderate agitation. The solubilization of the type-specific polysaccharide of *C. perfringens* Hobbs 5 by vigorous stirring at 100 C was based partially on this observation (2). It occurred to us that a low-temperature extraction might accomplish the same task and reduce cellular disruption. A comparison study of hot- and cold-water extraction showed that the former contained more nucleic acid and less protein than the latter but differed little in the polysaccharide yield. Therefore, the milder cold-water extraction procedure was used.

Ion-exchange chromatography, which was used effectively for the isolation of *C. perfringens* Hobbs 5 polysaccharide, was not applicable in this case because *C. perfringens* Hobbs 10 polysaccharide was bound irreversibly to Dowex 1. An alternate purification procedure was devised which called for the precipitation of nucleic acid with 1% CTAB-0.2 M NaCl from a sample of polysaccharide which had been previously deproteinized with chloroform-butanol. The efficiency of these steps was evident as witnessed by the percentage of increase in the hexosamine content.

The final purification step took advantage of the high apparent molecular weight of the type-specific polysaccharide in gel permeation chromatography. The polysaccharide was excluded from Bio-Gel A-5m agarose with an exclusion limit of 5 million, though its molecular weight determined by ultracentrifugation was only 40,000. The remaining impurities were included in the gel matrix, whereas the polysaccharide appeared in the void volume. Comparison of the initial alcohol precipitate with that of agarose fraction I showed an approximate five-fold overall increase in purity based on hexosamine and a constant carbohydrate composition except for the disappearance of ribose.

TABLE 2. *Analytical composition of capsular polysaccharide*

Polysaccharide component	Percent by wt	Molar ratio
Glucose	29.0	4.1
Galactose	35.2	5.0
Galactosamine	12.0	1.7
Iduronic acid	7.0	1

Agarose fraction I gave a single precipitation line against *C. perfringens* Hobbs 10 antiserum and did not react with *C. perfringens* Hobbs 5 antiserum. This was considered a criterion for immunochemical purity. Agarose fractions II and III reacted weakly to both antisera. These results illustrated that the agarose column effected the separation of the type- and group-specific substances. Chromatography of agarose fraction I on DEAE-Sephadex with a linear gradient of NaCl, which resulted in the elution of a single polysaccharide fraction, indicated the high degree of purity of the isolated polysaccharide.

The direct separation of nucleic acid from the deproteinized polysaccharide fraction using gel permeation chromatography was attempted. This approach was not adopted, since a significant quantity of nucleic acid remained associated with the polysaccharide at salt concentrations up to 2 M.

The physical properties of agarose fraction I have not been fully investigated. However, the exclusion observed on Bio-Gel A-5m agarose and the formation of gels at concentrations approaching 5% (unpublished observation) allude to the possibility that the type-specific polysaccharide is a highly extended molecule.

The tentative identification of iduronic acid as a substituent of a purified type-specific bacterial capsular polysaccharide was an unexpected result, considering its limited distribution in nature. The iduronic acid content is probably greater than reported, considering the ease with which the compound is destroyed under hydrolytic conditions. The purified and immunologically homogeneous polysaccharide isolated from *C. perfringens* Hobbs 10 was composed of glucose, galactose, galactosamine, and iduronic acid in a molar ratio of 4.1:5:1.7:1, respectively. These substituents represented 83% of the dry weight of the polysaccharide; a trace of peptide material was not

removed by the purification procedure. The low quantities and variable composition between samples did not permit study of the peptide portion at this time.

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